

## Response to Reviewer 1 Comments.

We would like to thank the reviewer for their constructive feedback and suggestions. We present our responses and the resultant changes to the original manuscript below. We have re-numbered the reviewers' comments (R1 or R2 C#1, C#2 etc.) and split some up (a, b, c etc.) in order to respond to individual points. Our responses are written below in blue 10pt text with changes indicated by bold type.

### General comments:

The authors heated 20 samples of minerals (K-feldspar, plagioclase feldspar, quartz, clay, dust surrogate and carbonate) as well as 4 samples of biological material (Snowmax, lichen, birch pollen washing water and cellulose) using two different heat test procedures. The first was a wet heat test where the samples were submerged in a bath of boiling water and the second was a dry heat test at 250 °C in an oven for 4h. The authors then measured the samples before and after the two types of heat tests and display their results as frozen fractions, as box plots and as n\_s plots. The authors then have rather long discussions of speculations (the word assume/assumption is found at least 10 times in the manuscript, at times justified and at times not, when the assumption could be resolved with further experiments). They speculate about what could have driven the differences before and after heat for the different heat tests and for the different types of samples. In general, the authors reference the literature adequately and thoroughly.

The research question is certainly worthwhile, and the authors' systematic approach is a good idea for evaluating the general applicability and the interpretation of a change in INA after a heat test. I commend the authors for approaching this problem systematically. However, this manuscript is currently too preliminary to be published. This study can be made significantly stronger to make an impact on the community and for the work to be built upon in the future. My key recommendations to improve the study before publication are below:

**R1C#1.** (most important recommendation) The authors are missing key experiments for further conclusions to be drawn. Specifically, the authors should run all 20 mineral and all 4 biological samples in a comparable dry heat test at 95 °C in an oven for 30 mins (or the same amount of time the sample was submerged in the water bath). This test is necessary, since the authors make many assumptions of what can be the cause of inconsistencies between their wet heat test and their dry heat test. Yet, comparisons have 3 variables being changed in both sets of heat tests: the method, the temperature and the time of heating. I would also encourage the authors to consider re-running their dry heat test at 30 mins (or running all their heat tests at 4h), which would add additional columns in Table 3. These additional experiments would really strengthen the systematic approach that the authors are attempting to present in this work. Right now I am left wondering what is the effect of wet vs dry and what is the effect of temperature and what is the effect of heating time?

There are effectively four variables we have investigated here: mode of heating (wet vs dry), type of INP, duration and temperature and so have tried to strike balance between all four of these. The heating conditions we used for both wet and dry modes were chosen on the basis of being representative of those typically used in the literature (refer to Table 1 in the manuscript and paragraph directly below). Instead of varying the heating conditions we chose to test a large array of

mineral samples due to the diversity of atmospherically relevant minerals groups and the variability of INA seen between samples of the same mineral type. Time and resources limit us to a finite number of tests we can carry out and as such the full scope of experiments suggested within R1C#1 is unrealistic - around 50 additional droplet freezing assay runs from this comment alone. Furthermore, the key objective of this study was to test the validity of the key assumption made throughout the community that the INA of minerals is insensitive to heat and the experiments we presented are well-suited to doing achieving this goal.

However R1 makes a valid criticism that we contrasted the different heating modes (wet/dry) at different temperatures and durations. **We therefore carried out further heat tests on a selection of samples – BCS-376 Microcline, Snomax and Birch pollen washing water: Dry heat at 95 °C for 30 min, 95 °C for 4 h, 250 °C for 30 min and wet heat at 95 °C for 4 h. These new results are shown in Fig B2 in Appendix B.**

We made the reasonable assumption that samples that did not deactivate after dry heating at 250 °C for 4 h would also not deactivate if dry-heated at a lower temperature and/or for a shorter duration. Therefore, we did not perform extra dry heat tests on other mineral groups such as quartz, plagioclase and the clays. **An additional wet heat test on Fluka Quartz at 95 °C for 4 h is shown in fig 4b.** We also point out that 4 h/95 °C wet heat tests were in the original manuscript for ATD (fig 4b, now 6b) and BCS-376 Microcline (fig 2b).

Overall, this allowed us to conclude that heating duration is a slightly more important variable when wet heating than in dry heating where it appears secondary in importance to temperature. Also, Snomax, and to a lesser extent BCS-376 microcline, showed differences in responses to being heated wet and heated dry for the same duration and temperature. These extra tests, and the assumption that samples resistant to dry heat at 250 °C are also resistant at 100 °C, allowed us to **add a column to Table 3 in Section 4 showing the characteristic responses to dry heat tests at lower temperature of 100 °C and also allowed us to refine our conclusions and recommendations sections.**

**R1C#2a.** Furthermore, there are important background water tests missing in this manuscript. The authors should address these details thoroughly before publication. In general, daily blank tests discussed in lines 261-262 are not experiment controls, and do not represent adequately the experimental procedure each sample is submitted to. The authors use 0.1 µm pre-filtered, cell culture-grade deionised water, and could the authors show the following background water data:

A sample of background water that was heated in the same type of vial as with the wet heat test (line 208).

A sample of background water that was heated in the oven at 95 °C in an oven for 30 mins.

A sample of background water that was passed through the same experimental procedure following the sample after heating at 250 °C in an oven for 4h.

**A new plot ( $f_{ice}$  vs T) with handling blanks and five background water tests is shown in Fig S3 in the Supplementary Info.**

**R1C#2b.** A sample of background water that passed through the nylon net filter and the cellulose acetate filters (referred to in line 192). I highly suspect that cellulose acetate filters leach material. These control frozen fractions must be shown. Show the data discussed in lines 221-224.

We are grateful for R1 for suggesting this because it revealed suspected contamination of the stainless steel filter holder (Advantec 301000) we used with the nylon net filter from the birch pollen washing water (BPWW). We believe this is the case as i) The handling blank we did using the same procedure produces a steep signal at around  $-19\text{ }^{\circ}\text{C}$  but did not before the original experiments. This is despite thorough washing of all parts (PTFE gaskets, metal screens) in isopropanol and deionised water. ii) Handling blanks of the nylon net and cellulose acetate filters (CA) used separately did not produce this steep signal.

For this reason, we adapted the filtration method in section 2.2 and repeated all experiments with birch pollen washing water and lichen using a protocol which did not involve the stainless steel filter holder. We did this by taking the raw suspensions from the vial with a sterile 1 mL syringe and then pushing them through a disposable  $0.2\text{ }\mu\text{m}$  cellulose acetate filter (Sartorius Minisart). The raw suspensions were pushed through with no apparent resistance and resulting filtrates were crystal clear, suggesting the  $10\text{ }\mu\text{m}$  nylon net filter was not needed. The handling blank for this process (Fig S3) shows the CA filter raised the background slightly but this was avoided by pre-flushing it with 10 mL of deionised water.

**As a result of these adaptations, we found that the INA of the BPWW and lichen extracts are indeed reduced to background levels by the dry heating ( $250\text{ }^{\circ}\text{C}$ ), in line with previous works (Pummer et al., 2012). This allowed us to update the figures and conclusions accordingly, as well as the summary in Table 3.**

**R1C#2c.** What is the role of the pre-sterilized dry heating at a different temperature? Shouldn't the procedure also involve the same  $250\text{ }^{\circ}\text{C}$  temperature as the experiment?

This was following a general protocol for sterilising glassware stored in ambient air, rather than one for specifically destroying contaminating INP. **A dry-heating handling blank at  $250\text{ }^{\circ}\text{C}$  is shown in Fig S3** and this shows that this treatment reduces the background somewhat compared to the blanks 1-5 which were pre-sterilised at  $175\text{ }^{\circ}\text{C}$ . However, the vast majority of experiments produced data well above this background anyway so pre-heating at  $250\text{ }^{\circ}\text{C}$  would have been only marginally beneficial.

**R1C#3.** The wet heat test will certainly have the water evaporate and therefore change the concentration of the material within the solution. How are the authors accounting for changed in concentration of the ice active material? relevant to the discussion in lines 145-147.

Refer to Section 2.3.1 (Wet heating) "The vessels were sealed tightly to prevent the evaporation of water from the vessel causing an increase in concentration of the suspensions". We tested this by weighing vessels before and after a wet heat run and the mass was unchanged within 0.1g. We also ensured the water bath was never high enough to reach near the lid and potentially leak into the vessel.

**R1C#4a.** The authors should make every effort to compliment the study with alternative measurements. I can appreciate that substantially more work would be required, but it would allow the manuscript to be much more concise rather than listing a list of speculations (for example, the K-feldspar discussion spans pages 11-14 of speculations). For example, ideas presented in lines 365-377 could be address with elemental analysis and presence of N. Or with a protein test such as the Lowry method.

The text the referee refers to is not simply speculation. We think the majority of this is a robust discussion of the mechanistic reasons behind the observed heat test responses. For example, in the case of the hypothesis for the wet-heat deactivations of quartz and k-feldspar, the ideas are backed

up by our experiments and by existing literature. **We have removed the discussion on lines 379-387 of the original manuscript as it is the most speculative out of all the ideas presented, and have replaced this with a discussion about coatings blocking active sites on K-feldspars in light of findings by Pach et al (2021).**

Regarding suggestions for extra chemical analysis, we re-iterate that the core motivation was to empirically test the heat sensitivity of mineral samples. The further work that the referee suggests will form the basis for future studies.

**R1C#4b.** Ideas in lines 368-370 could be tested with a heat test at the same temperature as the wet test (see my point number 1).

See comments above (R1C#1)

**R1C#4c.** In addition, total mass of the material after both heat tests can be weighed and measured (assuming the wet heat test and be dried out).

**We did this for the dry heat tests (see Supplementary Table 1) and found that the change in mineral samples' mass did not correlate with dry-heat deactivation.** Weighing after wet-heating would be problematic as drying them would require, in-effect, dry-heating them.

**R1C#4d.** Have the authors attempted to dry their material and repeat the heat tests multiple times? Does a wet heat test followed by a dry heat test and vice versa have any effect?

We have not performed these tests and while they would be interesting supplementary experiments they do they do not fall within the primary goals of the study which is to evaluate the common heat tests methodology used..

**R1C#4e.** Evidence of chemical composition would also be particularly helpful, for example total organic carbon analysis, ion chromatography, elemental analysis, etc. I'll add here that my criticism is also a general one to our community where we tend to only show INA of material when these results cannot simply be compared without other measurements (such as surface area and/or composition).

The majority of mineral samples we used in our study have featured in previous studies about their ice-nucleating properties and as a result a wealth of compositional information is available in the literature. Using this we compiled values for specific surface area and compositional (purity) for most of our samples in Table 2, Section 2.1. We accept that some of these baseline properties may change after our heat treatments, but we reiterate this falls outside the scope of this study (which specifically INA responses to heat) but could form the basis of a future study focused on a smaller number of samples.

**R1C#4f.** Finally, point d) at lines 719-725, I would recommend that the authors either don't discuss it, or do the experiments.

**We accept this falls outside of the stated scope of the study and have removed this passage.**

**R1C#5.** The authors should further describe their detailed storage protocols. These protocols come up a few times as excuses for differences but should be detailed to teach the community exactly what was done. (Examples include Line 166, 195 and 201). I'll also add that the authors' justification on lines 234-235 is very good.

We have added “The biogenic samples were stored cold (Snomax at -20 °C, raw birch pollen and dried lichen at – 4° C) or at room temperature in the case of MCC and they were made to suitable concentrations according to existing literature protocols”

And “All mineral samples were stored at room temperature in darkness and suspensions were prepared by mixing 0.1 g of sample with 10 mL water in 20 mL borosilicate glass vials (Samco type T006/01, Surrey, UK).

**R1C#6a.** The authors use a high concentration of material (stated as 20 mg/mL on line 190).

We accept the concentrations we used were rather high in the case of Snomax, BPWW and MCC (see response to R2C## below) **so we have therefore re-done the experiments at maximum concentrations of 0.05%, 0.1% and 0.5%, respectively.** We found that Snomax did not show residual INA after a 30 min wet heat test and was more thoroughly deactivated. BPWW and MCC at lower concentrations did not appear to behave differently to the original experiments done at higher concentrations in terms of  $n_m(T)$  values or their heat responses.

**R1C#6b:** Are the authors working with suspensions or homogeneous solutions (line 230)?

Although we generally have referred to all samples as suspensions throughout, **we have clarified this point in the case of BPWW and lichen in section 2.2: “The birch pollen and lichen samples could not be immediately dispersed in water as they required additional filtration steps to produce visibly clear homogeneous extracts rather than particulate suspensions.”**

**R1C#6c.** This fact is important since after heating, material’s solubility can substantially be affected. How have the authors addressed a possible change in solubility before and after their heat tests? What is the effect of concentration on the heat test? I think a series with one type of mineral sample with different concentrations for both heat tests would be an interesting series to present.

We have addressed this by carrying out additional runs at varying concentrations in addition to their ‘standard’ concentrations, before and after both standard wet and dry heating for:  
BCS-376 microcline (0.1% and 0.02%)  
Fluka Quartz (2.5%, 0.1%)  
ATD (2.5%, 0.1%, 0.01%)  
NX Illite (2.5%, 0.1%)  
Snomax (0.002%)  
BPWW (0.02%, 0.002%)  
Lichen (0.5%)  
MCC (0.01%)

We then plotted the results in  $ns(T)$  form or  $nm(T)$  where appropriate in Fig B1a-h. In all cases, the  $ns(T)$  or  $nm(T)$  curves, both heated and non-heated are coherent indicating there is no concentration dependence. This is explained fully in Appendix B and referred to in the main text where appropriate. Note there is no data for dry heat tests for the biological samples as they were all already fully deactivated at the highest concentrations.

**R1C#7.** Can the authors show what the role of the time during the heat test can have? This information would be particularly helpful to determine and optimal temperature and time of heating for subsequent experiments by the community (and will be better cited).

This is addressed with new data presented in Figure B2 and discussed in the final paragraph of Appendix B, (see also R1C#1) where we performed heat treatments on a subsection of samples at varying temperatures and durations. The general conclusion was that when we varied heating duration, longer wet heat tests resulted in further deactivations whereas dry heat tests showed similar results regardless of duration.

**R1C#8.** Have the authors attempted to combine one of their mineral samples with one of their biological samples? This test would better represent an ambient measurement and see if the effect is cumulative or not (see (Steinke et al., 2020)).

This would be very interesting but to gain meaningful insight it would require several combinations of mineral and biological samples with differing heat resistances, heated both wet and dry. We believe this would be better placed in a further study where it could be compared with natural samples such as fertile and desert soils.

We have added “Ideally, this should be conducted using a combination of model materials like we used in this study but also natural materials such as fertile soils, desert dusts, surface waters and precipitation samples. Also, the effects and suitability of alternative dry heat protocols on aerosol filters should be investigated” to acknowledge this point.

**R1C#9.** References: In general, Table S1 could be included in the main text as a reader-friendly reference guide for future work to compare and built upon. Good job to the authors for this compilation - although mentioned in the text, did the authors want to also include (Bogler and Borduas-Dedekind, 2020) in their table S1?

We have moved this table to the main text (Table 1) and updated it to include Hiranuma et al. (2021), Zinke et al. (2021) and also Tobo et al. (2014) which was omitted. The table is intended to highlight the heat treatments used for samples collected in the environment rather than heat resistance of particular ice-nucleating materials which is summarised in Section 1, paragraph below Table 1. Bogler and Borduas-Dedekind (2020) was therefore not included as they did not perform their heat tests on environmental samples, rather on reagent grade lignin.

**R1C#10.** Title: Seems to me that the second part of the title is most relevant to the content of the work. I can encourage the authors to consider a title along the lines: Testing (or systematic evaluation) of the heat test for ice-nucleating ability of minerals and biological materials.

Changed to “An evaluation of the heat test for the ice-nucleating ability of minerals and biological material”.

**R1C#11.** The introduction can be substantially shorted to focus only on the heat tests experiments listed in Table S1.

Lines 74 –90 of the original manuscript have been condensed to the following in paragraph 7 of Section 1 (Introduction):

“While techniques such as genomic sequencing (Garcia et al., 2012; Huffman et al., 2013; Hill et al., 2014; Christner et al., 2008) and microscopy (Huffman et al., 2013; Sanchez-Marroquin et al., 2021) can reveal the presence of biological species in an aerosol sample that has been found to contain INPs, it remains difficult to characterise the ice-nucleating ability of these species over other constituents (e.g. mineral dusts) when a sample’s INA is analysed by, for example, a droplet freezing assay alone.”



**R1C#12.** Following all these comments and suggestions, can the authors create a recommendation rubric for heat test measurements: type (wet vs dry), temperature, length of time, with the ultimate goal to streamline how our community runs these heat tests in the future (including with and without hydrogen peroxide as some groups have done.)

While developing a robust heat test protocol is the overarching aim, it is necessary to break this down into achievable steps. The first was to test the assumption that minerals are not sensitive to heat. We have shown that they are and have provided the motivation to improve heat test protocols. At the end of Section 4 we detail improvements and caveats to using heat tests based on our findings. For example we offer recommendations to adapt the wet-heat test to avoid mineral deactivations. ) and recommend control tests on known INP standards.. Also, as a result of the additional work that R1 has suggested we have concluded the following about dry heating:

**“Our data shows that dry heating at a lower temperature of 95 °C preserved the activity of K-feldspar, however, it did not deactivate heat-resistant polysaccharide INPs and denatured proteinaceous INPs (Snomax) far less than when heated at the same temperature in water. This means that, for detection of proteinaceous INPs, dry heating at 95 °C holds no advantage over wet heating at 95 °C.”**

I'll just add a comment here to the authors, that I am very conscious of the additional efforts being requested for the revisions of this paper. Nonetheless, I think the authors and readers (including myself) would greatly benefit from the study being expanded in its conclusions and implications of the heat tests. I hope the authors will be encouraged to improve their work and go those extra steps further for the benefit of our community.

To summarise, we sadly did not have the time and resources to carry out all of the experiments suggested by R1 and we believe many of them are more appropriate for a follow up study. However, their comments were extremely helpful in expanding the conclusions and particularly for identifying the issue with the filtration equipment (R1C##).